ULTRASTRUCTURAL CHARACTERISTICS OF HUMAN OOCYTE MATURATION IN OVARIAN-STIMULATED CYCLES

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ABSTRACT

The material consisted of 73 oocytes obtained at follicle aspiration in ovarian stimulated women. Oocytes in various stages of maturation were either immediately fixed or cultured before fixation. Cytoplasmic changes which were regarded as being related to maturation were: a decrease in the number of vacuoles and multivesicular bodies, an increase in volume of the endoplasmic reticulum, clustering of mitochondria and the appearance of aggregates of tubuli of the smooth endoplasmic reticulum surrounded by mitochondria and mitochondria-vesicle complexes.

Certain unusual morphological features were found to cause misinterpretations at examination of oocytes in a light microscope. For instance, polar bodies can be mimicked by corona cells in the perivitelline space and pronuclei in oocytes by large mitochondria-vesicle complexes in the ooplasm. Further, ultrastructural studies have shown oocytes to be degenerating even though inspection at recovery did not disclose anything pathological.

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INDTRODUCTION

Oocyte maturation comprises: breakdown of the germinal vesicle (GV) membrane, formation of the first meiotic spindle, extrusion of the first polar body, formation of the second meiotic spindle, and arrest of meiosis (1). At this stage the oocyte is ovulated. Ovulation occurs about 36 hours after oocyte maturation commenced (2). The fertilizable life span of the ovulated human oocyte is not known but probably measurable in hours like in other mammalian ova (3). If the oocyte is fertilized, meiosis is resumed, the second polar body is extruded and the female and male pronuclei are formed.

Various maturation stages of human oocytes, obtained from ovarian wedges or by aspirating ovarian follicles - or in a few cases, obtained from the Fallopian tube - have been examined in the electron microscope (4-10). Maturation of oocytes in vitro has also been studied (11-14). Maturation in vivo is characterized by a general increase in the number of cytoplasmic organelles. In the resting oocyte of the primordial follicle, organelles are located juxtanuclearly. Golgi complexes, mitochondria and profiles of the smooth endoplasmic reticulum (SER) are not prominent whereas annulate lamellae are rather common. In maturing oocytes the converse is found and the organelles are distributed throughout the ooplasm. Moreover, microvilli develop on the cell membrane and cortical granules appear. In vitro matured oocytes show essentially the same changes during maturation as do oocytes developed in vivo. However, cultured oocytes lack a well developed Golgi apparatus, but have acquired spheroidal aggregates of tubuli of SER (11). The number of cortical granules increased markedly in preovulatory oocytes incubated for 3 to 6 hours after collection (15).

The clinical work carried out on human in vitro fertilization (IVF) and egg replacement (ER) has made studies on the maturation of human oocytes easier in many ways, thanks to, for instance, refinements of the techniques for ovarian stimulation, oocyte collection, and oocyte culture.

Oocytes obtained at follicle puncture are classified clinically as immature, intermediate, or pre-ovulatory (16). By definition, a fertilizable oocyte has extruded its first polar body. However, a polar body cannot easily be observed in the light microscope as cumulus corona cells surrounding the oocyte obscure the view. The exact stage of maturation of an oocyte at the time of incubation with spermatozoa is therefore usually not known. No exact and readily observable clinical sign or test has yet been presented by which to

tell the optimal time when an oocyte should be incubated with spermatozoa. In a clinical study it was found that incubation of preovulatory oocytes for 5 hours before adding spermatozoa enhanced the fertilization rate (17). Yet, not all oocytes classified as preovulatory become fertilized. Failure of fertilization can be due to disorders of the sperm cells or of the oocyte, or to incomplete maturation or perhaps to "overmaturation" of oocytes by preincubation.

In an IVF-ER program, all preovulatory oocytes are submitted to fertilization attempts and only immature oocytes can be fixed immediately for electron microscopy. Preovulatory oocytes which have not been fertilized can also be taken for microscopy. In the present study, the ultrastructure of these types of immature and preovulatory oocytes was examined to ascertain what changes occur during oocyte maturation and to find out whether the cultured unfertilized oocytes differ in appearance from preovulatory oocytes which had been fixed immediately. Therefore, a few immediately fixed preovulatory oocytes, never intended for replacement, were used for comparison.

MATERIAL AND METHODS

The material consisted of 73 oocytes obtained from 58 ovarian-stimulated women admitted to the department because of infertility, due mainly to damage of the Fallopian tubes. For stimulation, the women were given clomiphene citrate and human chorionic gonadotropin (hCG) or in a few cases clomivid, human menopausal gonadotropin and hCG. Follicle aspiration was carried out at mid-cycle, 34 to 36 hours after the hCG injection. The size of the follicles varied, but all follicles that could be seen were aspirated. Follicular fluid volumes were measured and the fluids surveyed for oocytes within 5 min of follicle aspiration.

The clinical classification of collected ova was made by judging the amount, dispersion and viscosity of cumulus-corona cells surrounding the oocytes, as visualized in a stereomicroscope. Immature oocytes were either denuded or surrounded by a corona consisting of a few to several layers of cells, whereas intermediate oocytes were surrounded by a small or medium large moderately dispersed and viscous cumulus mass. Oocytes were classified as preovulatory when surrounded by a large, dispersed and viscous cumulus mass (16).

Some oocytes were fixed immediately. A few immature and intermediate oocytes were taken for culture and kept in the incubator for 5 hours or for 2-3 days before fixation. Amost all the preovulatory oocytes were preincubated for 5 hours, incubated with spermatozoa for 24 hours and then cultured for 1-2 days or in one case 7 days, before fixation (Table I).

Follicular fluid volumes in follicles yielding immature oocytes ranged from 0.1 to 2.0 ml and those yielding intermediate oocytes ranged from 1.0 to 3.5 ml. Follicles from which preovulatory oocytes were obtained varied considerably in content, ranging between 1 and 37 ml (mean 6 ml).

Preparation of spermatozoa, methods for fertilization and oocyte culture have been described in detail elsewhere (18). In this study some minor changes were made at incubation of the oocytes with spermatozoa and at oocyte culture. Thus, the number of spermatozoa at incubation was 500 000, while at culture, test tubes, without liquid paraffin to cover the medium, were used. The gas in the incubator was 5% O_2, 5% CO_2 and 90% N_2 and the medium used was Earle´s medium. Patient serum was added to the medium used for incubation with spermatozoa (10% serum) and culture (15% serum). Spermiograms were analysed from the sperm samples used for IVF and only two were classified as seriously pathological.

Estradiol-17β and progesterone in plasma obtained from the women on the days around oocyte collection were analysed by conventional radio--immunioassay. The day of ovulation was presumed to be the day after the peak in plasma estradiol-17β, coinciding with a rise in plasma progesterone (19). Accordingly, all preovulatory oocytes from women in whom plasma hormone levels had been analysed (all but 5), were collected on the presumed day of ovulation.

Fixation of the oocytes was done by placing them in a solution of 2.5% glutaraldehyde and culture medium without serum (room temperature, pH 7.4). Immediately fixed oocytes were examined in a light microscope at fixation. In oocytes surrounded by several layers of corona cells or by a cumulus mass, the general appearance of the ooplasm and the presence of a polar body were difficult to judge. Cultured oocytes were examined at fixation shortly after removal of remaining corona cells surrounding some oocytes. The general appearance of the cytoplasm and the presence of a polar body at fixation were noted.

Oocytes that were denuded or surrounded by only a few layers of corona cells at fixation could be judged and were regarded as atretic when displaying a dense retracted ooplasm. Normal-appearing oocytes displayed a homogeneous, non-retracted cytoplasm.

Preparation of the oocytes for TEM was in all cases performed in the same manner. Thus, after fixation for a few days, the oocytes were washed in phosphate buffer, post-fixed in 1% osmium tetroxide in phosphate buffer,

dehydrated in increasing concentrations of ethanol and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate. Sections, from one to four levels, from each specimen were examined.

Two kinds of degenerating oocyte were found at TEM. Oocytes were classified as atretic when displaying extensive vacuolization throughout the cytoplasm and a condensed ground substance. Oocytes showing numerous vacuoles in the centre of the cytoplasm were classified as pre-atretic. Apart from numerous vacuoles, these oocytes showed a well preserved cytoplasm.

The maturation stages of the oocytes and their condition at fixation are presented in Table I. Oocytes clinically classified as immature were of two kinds viz. oocytes possessing a GV and oocytes without a GV at serial sectioning. Oocytes without a GV were usually surrounded by several layers of cells at collection. Ten oocytes pocessed a germinal vesicle (GV). Among the cultured preovulatory oocytes, all the normal ones possessed a polar body.

RESULTS

General characteristics of normal oocytes judged by TEM

Immature oocytes were surrounded by corona cells which extended cytoplasmic projections deep into the zona pellucida (Fig. 1). In mature oocytes, these projections had been retracted. In one immature oocyte, a few corona cells were observed, trapped in the perivitelline space (Fig. 2). Numerous slender, 4-5-µm-long, microvilli extended from the surface of the cells.

The cytoplasm membrane possessed numerous, 1-2-µm-long, microvilli which projected into the narrow perivitelline space and often extended into the zona pellucida. A row of peripherally located microfilaments were seen in the villi when cut transversely. Type C virus-like particles, either budding from the microvilli or lying free in the perivitelline space, were observed in some oocytes, regardless of their stage of maturation.

The oocyte cytoplasmic ground substance consisted of a fine flocculent material throughout the ooplasm. Mitochondria were rounded, with a diameter of about 0.5 µm and contained a few, concentric inner membranes (Fig. 3). Golgi complexes, which were not so prominent, appeared scattered in the cytoplasm. Each Golgi had a size of about 0.5 x 1 µm and consisted of 4 to 6 membranes surrounded by small vesicles. Lysosomes, with a diameter of about 2 µm, were observed in many oocytes. Annulate lamellae were only occasionally seen in a few oocytes.

Immature oocytes possessing a germinal vesicle

The cytoplasm contained membrane-bound cortical granules, with a diameter of 0.3 to 0.5 µm, in varying numbers located close to the plasma membrane and deep within the cytoplasm. The mitochondria were evenly distributed, though clusters were occasionally seen peripherally. Usually, there was a close association between the mitochondria and SER which appeared as small irregular profiles or sometimes as flattened sacs with a size of about 0.5 to 1 µm. Some vacuoles with a diameter of 1-2 µm were observed in the centre of the oocytes. Flattened sacs of SER were often found in their vicinity. Multivesicular bodies were fairly numerous in the oocytes. These structures, which had a diameter of about 1 µm, contained several doughnut-shaped inclusions and occasionally one or two dark bodies (Fig. 4). In two oocytes, a few small aggregates of closely packed SER tubuli were noted.

The germinal vesicle, which was usually spherical and had a diameter of about 20 µm, was located centrally or, in a few oocytes, close to the cell membrane. In the latter oocytes, a cone-formed zone of cytoplasm with relatively few organelles was noted between the nucleus and the oocyte plasma membrane. The nuclear envelope, consisting of a double membrane, showed regular dense spots representing nuclear pores. A large nucleolus was observed in some nuclei with dark chromatin concentrated around it (Fig. 5a). Two oocytes cultured for 5 hours showed an irregular outline of their nuclear membranes (Fig. 5b).

Immature oocytes without a germinal vesicle

In immature oocytes, which were fixed immediately, the cytoplasmic organelles and their distribution were generally rather similar to those in oocytes in the GV stage. However, in some oocytes, cortical granules were observed only by the cell membrane and none deep in the cytoplasm (Fig. 6a). Some vacuoles were observed in most of the oocytes but multivesicular bodies were rarely seen. The close association between SER and mitochondria was even more pronounced than in oocytes possessing a GV. In areas where no SER was seen, mitochondria were sparse and where profiles of SER were common, mitochondria tended to cluster (Fig. 7). Moreover, SER in the form of flattened sacs was more common than in the GV stage. In two oocytes a few small aggregates of flattened tubuli of SER were observed. One of these oocytes also possessed a few (3 to 4) small mitochondria-vesicle (MV) complexes.

Intermediate oocytes

The general appearance and distribution of the cytoplasmic organelles in intermediate oocytes which were fixed immediately, resembled in the main that of preovulatory, immediately fixed oocytes. Multivesicular bodies were not seen in any of the oocytes. In one oocyte a chromosome was found close to the cell membrane. It also contained a few small MV complexes.

Preovulatory oocytes

In cultured preovulatory oocytes, cortical granules were usually (but not always) located close to the cell membrane in a single layer. Membrane-bound vacuoles were infrequent and multivesicular bodies were lacking. SER often appeared as distended rounded sacs with a diameter of about 1 to 3 µm, as aggregates of tubuli (Fig. 8), and as vesicles surrounded by mitochondria.

MV complexes were found in all the cultured oocytes. The complex consists of a single membrane-bound vesicle, containing a medium-dense flocculent substance, intimately surrounded by a rim of usually 5 to 20, occasionally more, rounded mitochondria (Fig. 9). No signs of communication between MV complexes were found. The number of complexes varied from a few to about fifty in sections from different oocytes (Fig. 10). Their size varied too, the smallest having a diameter of about 1.5 µm and the largest about 5 µm. In two cultured oocytes a few large MV complexes with a diameter of about 10 to 15 µm were observed (Fig. 11a).

In most cultured oocytes, more or less prominent aggregates of flattened tubuli of SER, usually surrounded by one to three layers of mitochondria, were observed (Figs. 9,10). The structures were about as large as MV complexes, and even unusually large aggregates with a diameter of 10 to 15 µm were observed. Occasionally, these structures were seen in close association with MV complexes. In some oocytes, both mitochondria-SER aggregates and MV complexes were numerous. In these oocytes, the conventional appearance of SER profiles was rare and almost all the mitochondria were clustered around the two types of structures (Fig. 10).

In preovulatory oocytes which were immediately fixed, aggregates of SER tubuli and MV complexes were few but otherwise no differences were observed between these oocytes and cultured preovulatory oocytes.

All three intermediate oocytes and one of the two immature oocytes cultured for 2-3 days resembled cultured preovulatory oocytes and showed several aggregates of mitochondria - SER and MV complexes.

Ultrastructure of degenerating oocytes

In atretic oocytes, microvilli on the cytoplasm membrane were sparse or lacking. Of the immediately fixed preovulatory oocytes, one was atretic. Another immediately fixed oocyte, which was classified as preovulatory at collection, possessed a GV and was found to be pre-atretic.

The oocyte fixed after 8 days showed numerous vacuoles in the centre of the cytoplasm and also one large pronuclear-like structure. Higher magnification revealed that the structure, which had a diameter of 25 µm, consisted of a single membrane-bound vesicle surrounded by mitochondria (Fig. 11b). Pre-atretic oocytes (Fig. 6b) showed intact microvilli on the cell membrane.

DISCUSSION

The stage of maturation of the oocytes at collection was judged clinically by the appearance of the surrounding cumulus-corona cell mass. However, it is often difficult to judge the exact stage of maturation of an oocyte when observing it in a light microscope. One oocyte, classified as preovulatory, even possessed a GV. All the cultured normal-appearing preovulatory oocytes possessed a polar body at fixation. However, since the exact stage of maturation at collection was not known, some oocytes might have extruded their polar body during the culture period.

The ultrastructure of cultured oocytes could have been influenced during culture. However, most showed good preservation at TEM and, from this point of view, culture for a few days does not seem to affect oocyte ultrastructure. The oocyte cultured for 8 days, however, showed numerous vacuoles and was regarded as degenerating. When comparing immediately fixed preovulatory oocytes with those which were cultured, the former were found to possess only a few aggregates of mitochondria - SER and MV complexes while the latter usually possessed several of these structures. Otherwise no ultrastructural differences were observed between the two groups of preovulatory oocytes.

There could be several reasons for fertilization failure in addition to a maturation factor of the oocyte, such as genetic disorders or inferior sperm quality. However, we have not yet found any qualitative ultrastructural differences in cultured oocytes distinguishing unfertilized from fertilized uncleaved oocytes which could indicate an ooplasmic component (20).

The cytoplasmic changes which were observed among the oocytes and which could be related to oocyte maturation were: a decreased number of vacuoles and multivesicular bodies, an increase in volume of the profiles of the endoplasmic reticulum, clustering of mitochondria, and the appearance of aggregates of tubuli of SER surrounded by mitochondria, and MV complexes.

The presence of numerous vacuoles probably indicates imminent atresia. Some vacuoles, however, were observed in immediately fixed preovulatory oocytes as well as in blastomeres of eggs in early cleavage stages (18). We therefore believe that a certain degree of vacuolization need not constitute a pathological sign. The function of these vacuoles, however, is still not known.

Multivesicular bodies were common in oocytes in the GV stage, but rare in later maturation stages. Multivesicular bodies belong to the lysosome system and probably have a digestive function (21), maybe at germinal vesicle breakdown.

The endoplasmic reticulum is observed as small irregular profiles early in maturation, but as circular profiles in preovulatory oocytes. The SER was closely associated with mitochondria and, in areas where SER was common mitochondria were also numerous. It might be that synthesis of products within the SER is energy demanding, or that mitochondria are provided with products from the SER. The shift in shape from a narrow to a circular profile implies an increase in volume of the reticulum, but it is not known what substance within the reticulum acounts for this increase. Since the reticulum is of the smooth variety, the change could be related to an increase in production of carbohydrates or lipids, or to production of some steroid hormones, or to calcium metabolism (21).

The distribution of mitochondria changed during maturation. Mitochondria were irregularly scattered in GV oocytes, clustered and closely associated with SER in immature oocytes without a GV, and arranged around spherical cisterna or rounded aggregates of tubuli of the endoplasmic reticulum in cultured pre-ovulatory oocytes. The presence of aggregates of mitochondria-SER has been described earlier (11), but their function is still not known. It seems that the aggregates appear before the formation of MV complexes and that the two types of structures belong to the same functional system. In cultured oocytes with many aggregates of mitochondria-SER, MV complexes were usually also frequent, and vice versa.

MV complexes have been found in early cleavage stages, but they rapidly diminished in number during cleavage (18). The functional implication of the

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complexes is as yet unknown, but it can be assumed that the mitochondria cooperate with the vesicles of the endoplasmic reticulum in producing some substance(s) which have a function related to fertilization and/or early cleavage. The complexes could be required at, for instance, the activation of the oocyte at fertilization, by releasing calcium ions into the cytoplasm (22). The number of complexes varies among the oocytes, the greatest number and also the largest complexes being observed in some cultured oocytes. A few unusually large MV complexes were observed in some cultured oocytes. These complexes could mistakenly be interpreted as pronuclei in the light microscope. Since all oocytes submitted to IVF are incubated for 5 hours before the fertilization attempt, it might be that those which are fertilizable at recovery get "overmatured" after incubation and that an abundance of MV complexes is a sign of "overmaturation".

Cortical granules have been found to increase in number during maturation and to be concentrated at the cell membrane in mature oocytes (15). In this study, however, even some immediately fixed immature oocytes possessed cortical granules only by the cell membrane and none deep within the cytoplasm, at examination of several randomly taken sections from each of the oocytes. Thus, the location of the cortical granules was not always a reliable indicator of the maturation stage of the oocyte.

Structures similar to budding and free-lying type C virus particles have been observed in human oocytes (24). The present study has shown that the particles appear at all stages of oocyte maturation. Since we have identified reverse transcriptase, which is a virus-coded enzym, and virus protein (p30) in follicular fluids (25), we believe that the particles observed are retroviruses. The reason for the seemingly irregular expression of virus particles among oocytes in different stages of maturation could be that synthesis of the particles is restricted to a certain stage(s) in the first and second meiosis, like the synthesis of Rous sarcoma virus during cell mitosis (26). Further experiments will be necessary to clarify this point.

Corona cells were occasionally seen inside the zona pellucida and might be taken for extruded polar bodies (12) or cytoplasmic fragments in the light microscope. These cells were observed to have numerous long projections differing from the small microvilli on the cells of the corona radiata. Since rat granulosa cells are known to form long projections when exposed to, for instance, prostaglandins or gonadotropins (27), it might be that the perivitelline space contains a high concentration of a substance which is

capable of affecting the appearance also of human granulosa cells.

Degenerating oocytes were of two kinds, viz. atretic oocytes and pre-atretic oocytes. Immediately fixed immature oocytes which were atretic had probably degenerated already early in the cycle whereas pre-atretic immature oocytes might have commenced degeneration shortly before collection. If this is true, approximately 20% of the immature oocytes had commenced degeneration shortly before collection. This is in agreement with earlier observations (28,29).

Since preovulatory oocytes cultured for 2 to 3 days were either normal or atretic, it is possible that degeneration of these latter oocytes had started already prior to collection. This view is strengthened by the fact that one preovulatory oocyte (clinically classified) was atretic at collection. Furthermore, another preovulatory oocyte (clinically classified) was pre-atretic and possessed a GV at recovery.

Some clinical conclusions can be drawn from the results. For instance, immature oocytes which showed a normal appearance in the light microscope had commenced atresia in approximately 20% of the oocytes, according to TEM. Thus, four out of five such oocytes might have the ability to mature in vitro. Among the preovulatory oocytes, one of four was atretic after 2 to 3 days of culture. However, degeneration might have started already prior to collection. The clinical classification of oocytes by their cumulus mass can be erroneous, since a preovulatory cumulus mass can harbour an atretic oocyte and a degenerating oocyte possessing a GV. Also the interpretation of pronuclei and polar bodies/cytoplasmic fragments can be erroneous in the light microscope. Thus, these structures can be large MV complexes or corona cells trapped in the perivitelline space, respectively. Consequently, not all oocytes in which pronuclei-like and polar body-like structures are observed are necessarily fertilized oocytes.

Table I. Time from oocyte recovery to fixation

Stage of the oocytes at fixation		0 hours	5 hours	2-3 days
Immature with a GV (N = 10)	N	6	2	-
	A	-	-	-
	PA	1[a]	1	-
Immature without a GV (N = 25)	N	12	2	2[b]
	A	5	-	-
	PA	4	-	-
Intermediate (N = 7)	N	3	-	3[b]
	A	-	-	-
	PA	1	-	-
Preovulatory (N = 31)	N	5	-	18
	A	1	-	7[cd]
	PA	-	-	-
Total, N		38	5	30

N: Normal
A: Atretic
PA: Pre-atretic

[a] Oocyte clinically classified as preovulatory.
[b] One immature and two intermediate oocytes each possessing a polar body at fixation.
[c] One oocyte showed several fragments and a well preserved cytoplasm in each fragment.
[d] Oocyte fixed 8 days after recovery.

LEGENDS

Fig. 1. Immediately fixed immature oocyte. Cytoplasmic projections from the corona radiata cells are extending into the zona pellucida. The perivitelline space is narrow and numerous microvilli are projecting into this space. Cortical granules are located by the cell membrane and deep in the cytoplasm. Mitochondria are scattered in the cytoplasm. A few vacuoles are observed. x3000.

Fig. 2. Immediately fixed immature oocyte. A few corona cells with long and slender microvilli are observed in the perivitelline space in this oocyte. x2000.

Fig. 3. Rounded mitochondria with a few peripherally located crista. Profiles of SER are seen in close association with some of the mitochondria. x100 000.

Fig. 4. Immediately fixed oocyte in the GV stage. Several multivesicular bodies are observed in the cytoplasm. The mitochondria are rounded and contain few concentric crista. The mitochondria are scattered in the cytoplasm but usually associated with SER which appears as small irregular profiles and as small sacs. A small Golgi complex and a few dark cortical granula are seen. x26 000.

Fig. 5. (a). Immediately fixed oocyte in the GV stage. The nucleus is spherical and contains a dark nucleolus surrounded by chromatin. The nuclear envelope is a double membrane and dark spots represent nuclear pores. x12 000.

(b). Cultured oocyte in the GV stage. The nuclear membrane has an irregular course. x28 000.

Fig. 6. (a). Immediately fixed oocyte without a GV. Cortical granules are located only by the cell membrane. Mitochondria are evenly distributed throughout the ooplasm. A few vacuoles are seen. x1500.

(b). Immediately fixed immature oocyte with numerous vacuoles. x2100.

Fig. 7. Immediately fixed immature oocyte without a GV. There is close association between SER and mitochondria. Where SER is common, mitochondria tend to aggregate. Cortical granules are located by the cell membrane and deep in the cytoplasm. Microvilli are seen on the cell surface. x27 000.

Fig. 8. Cultured preovulatory oocyte. SER appeared as distended rounded sacs or was organized in a tubular system. x18 000.

Fig. 9. Cultured preovulatory oocyte. MV complexes and aggregates of SER and mitochondria are commonly observed in cultured oocytes. x17 000.

Fig. 10. Same oocyte as in Fig. 9. Numerous MV complexes and aggregates of SER-mitochondria are observed. Almost all the mitochondria are associated with these structures. x2600.

Fig. 11. (a). Cultured preovulatory oocyte. A few pronuclei-like structures were observed in this oocyte. x2200.

(b). Preovulatory oocyte cultured for 8 days. A large MV complex is seen. x4500.

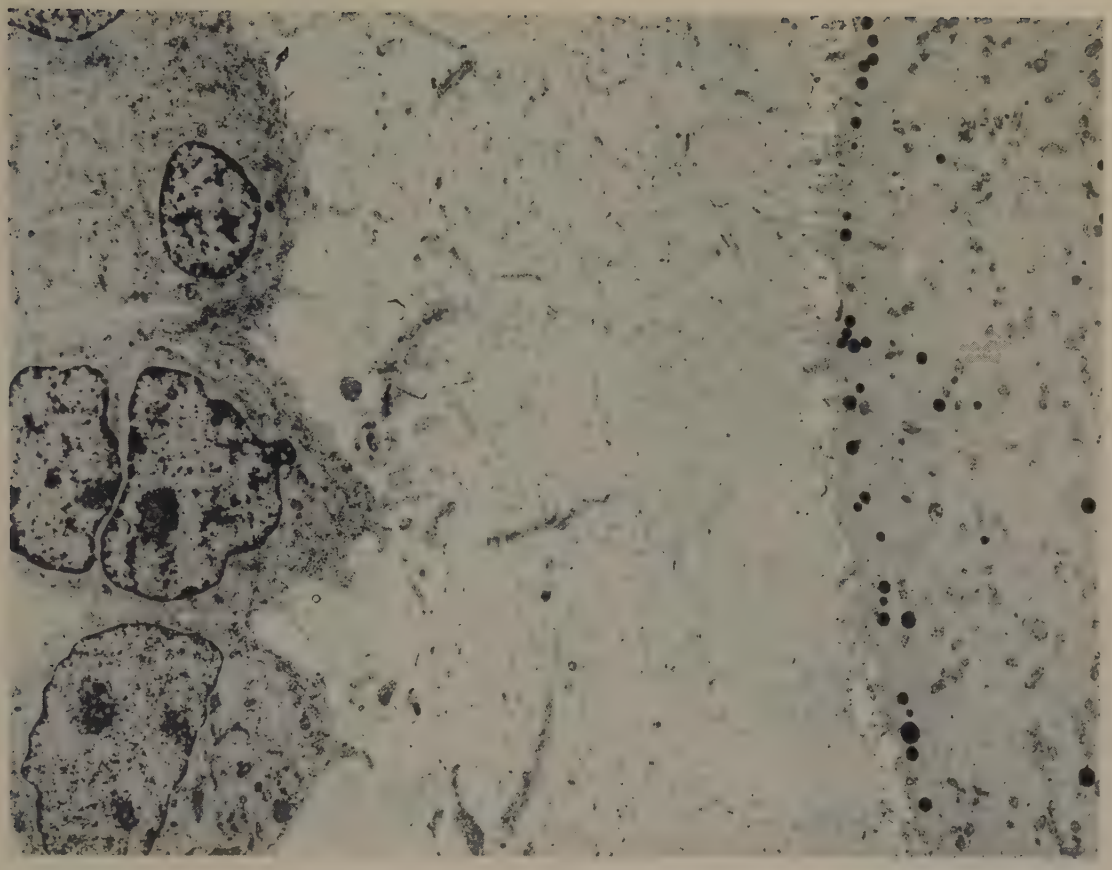

Fig. 1

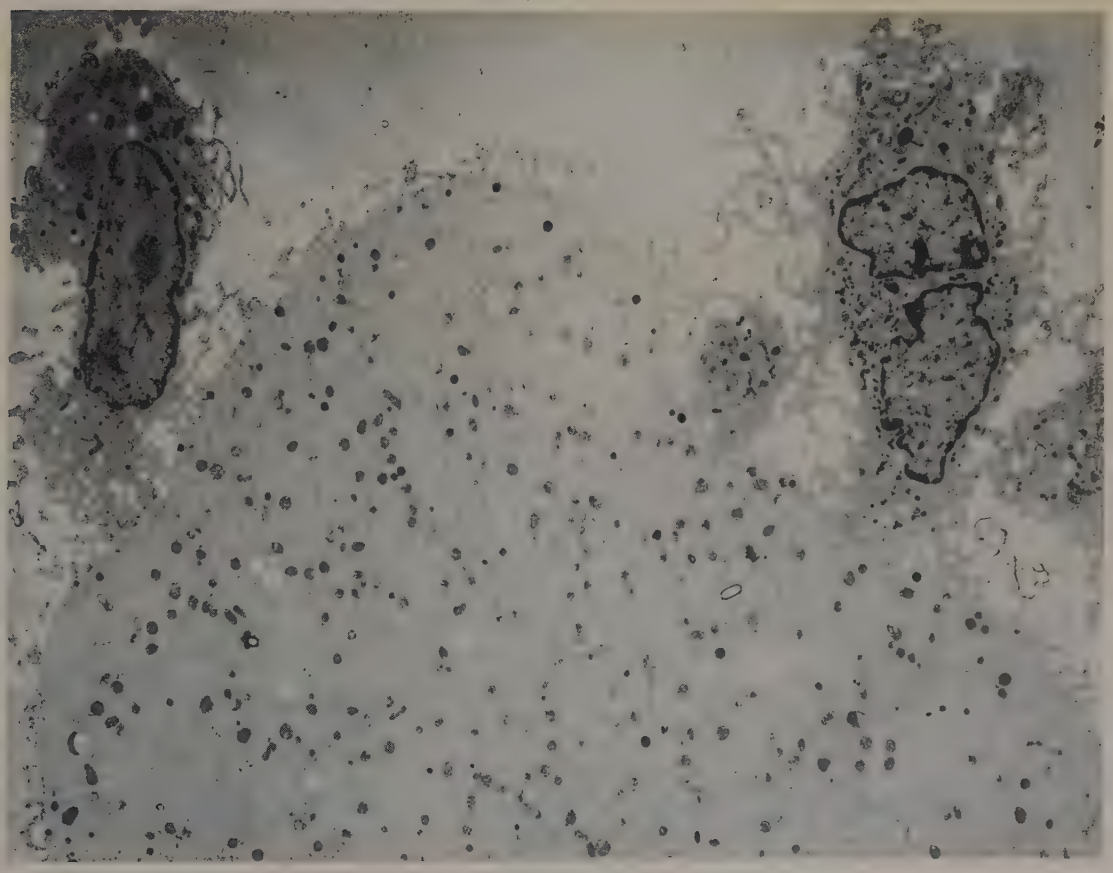

Fig. 2

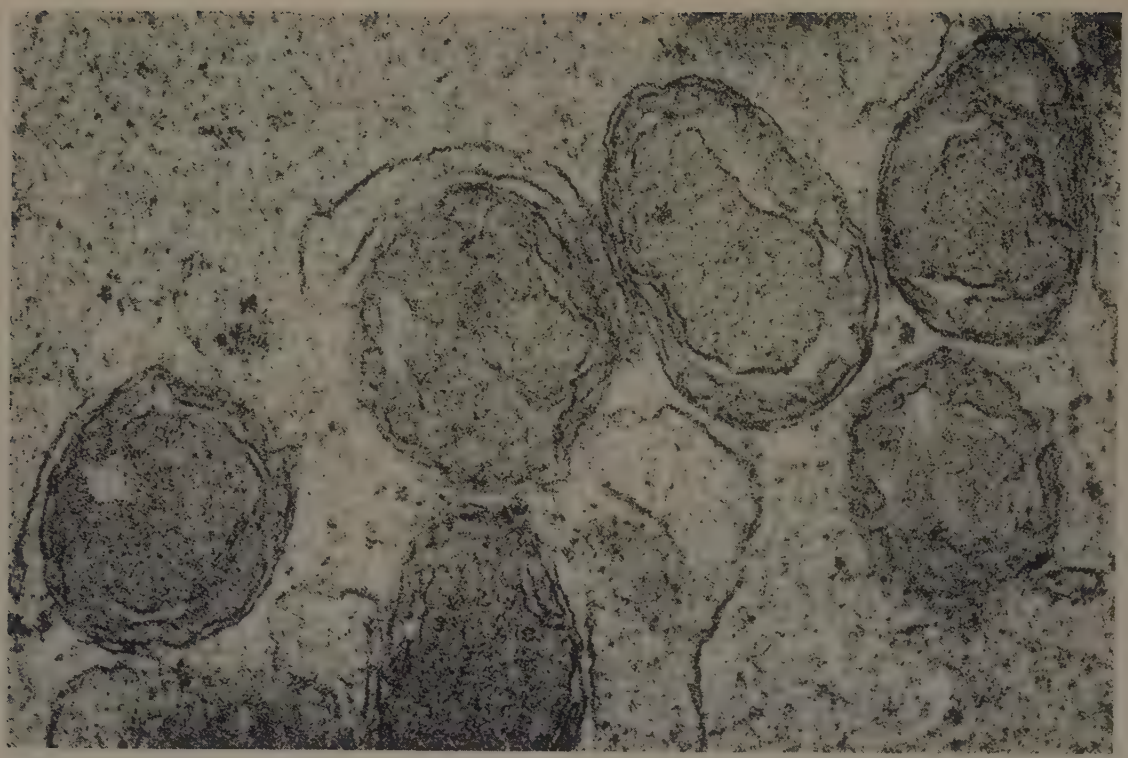

Fig. 3

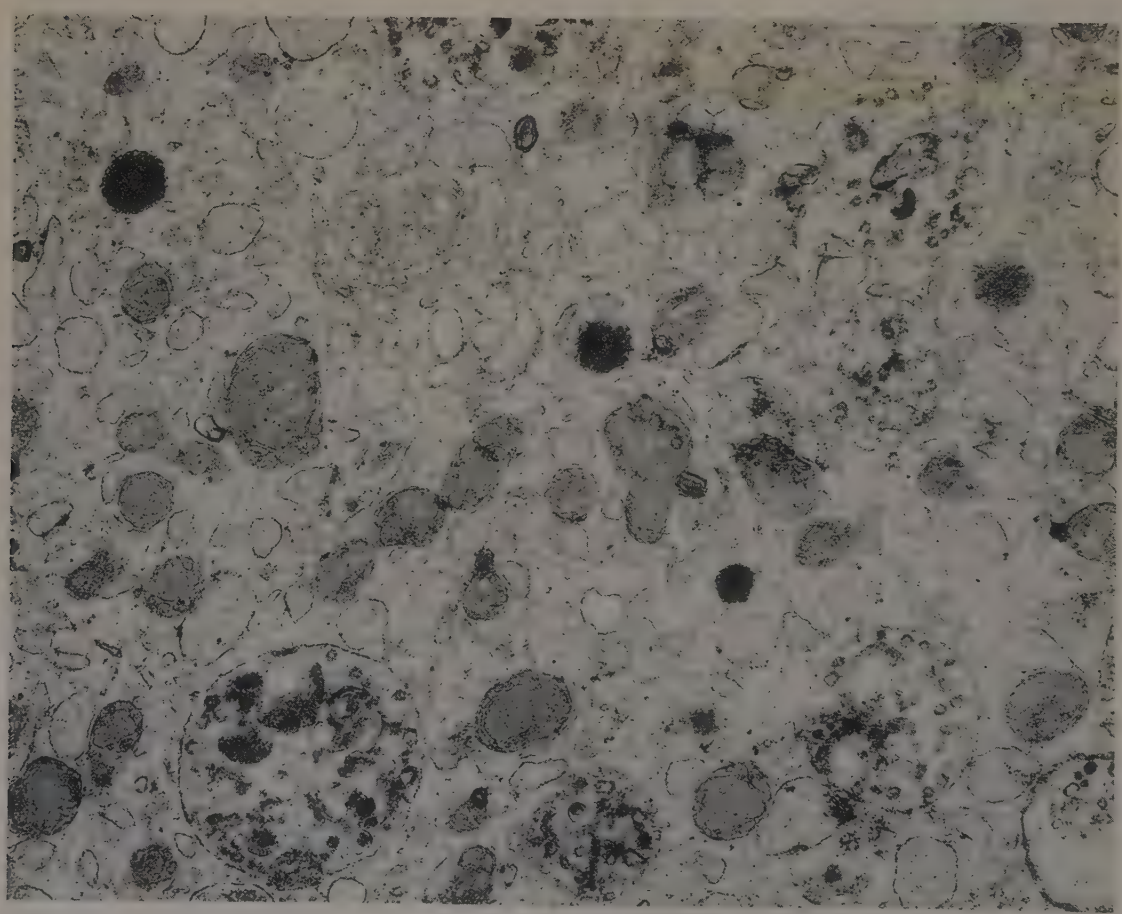

Fig. 4

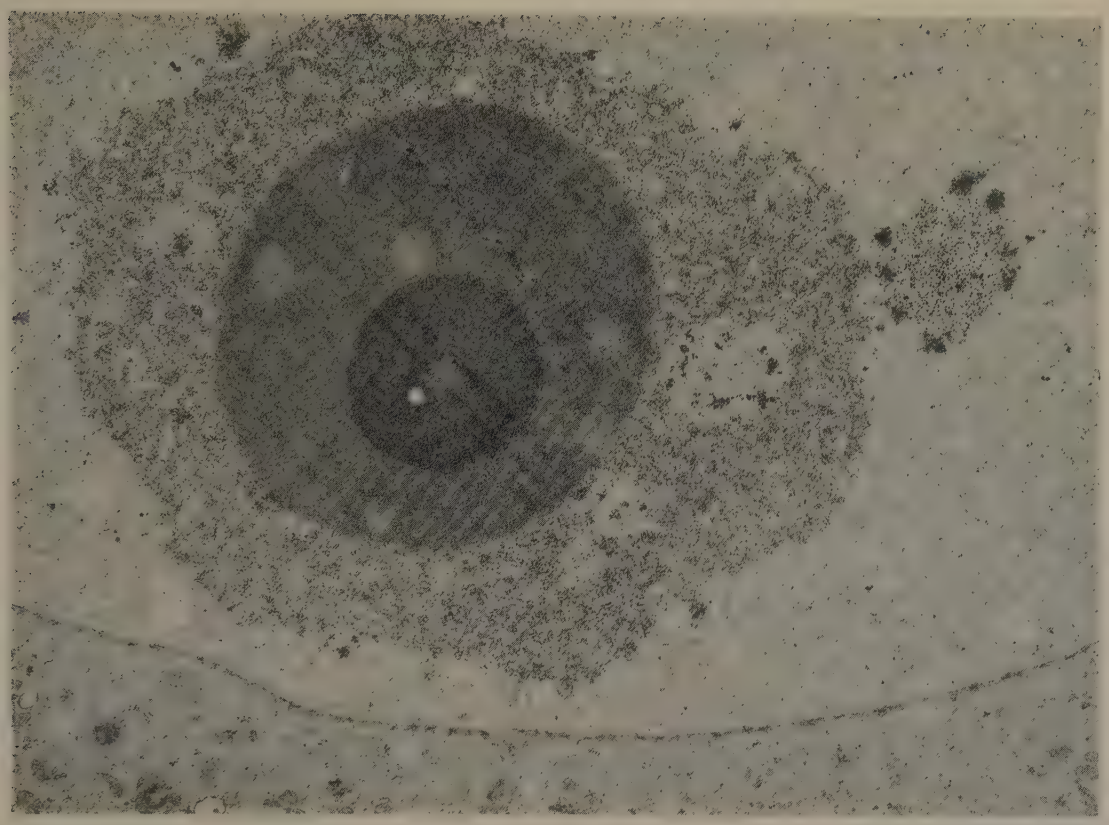

Fig. 5a

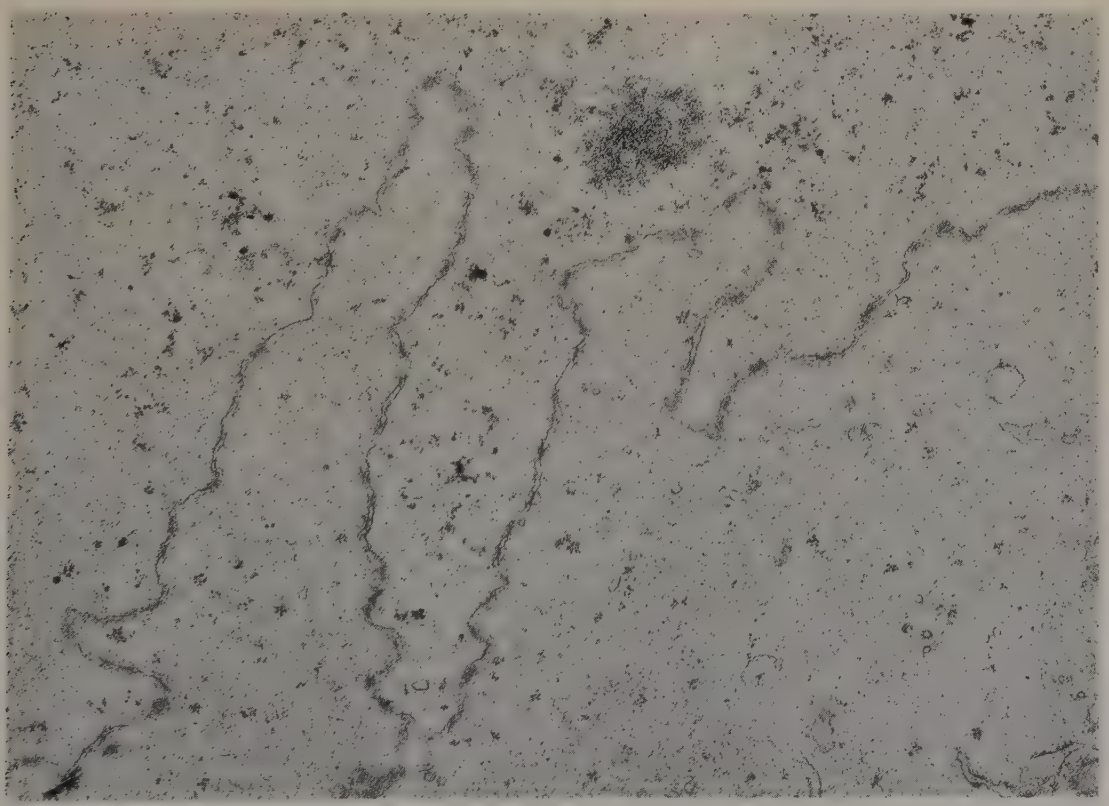

Fig. 5b

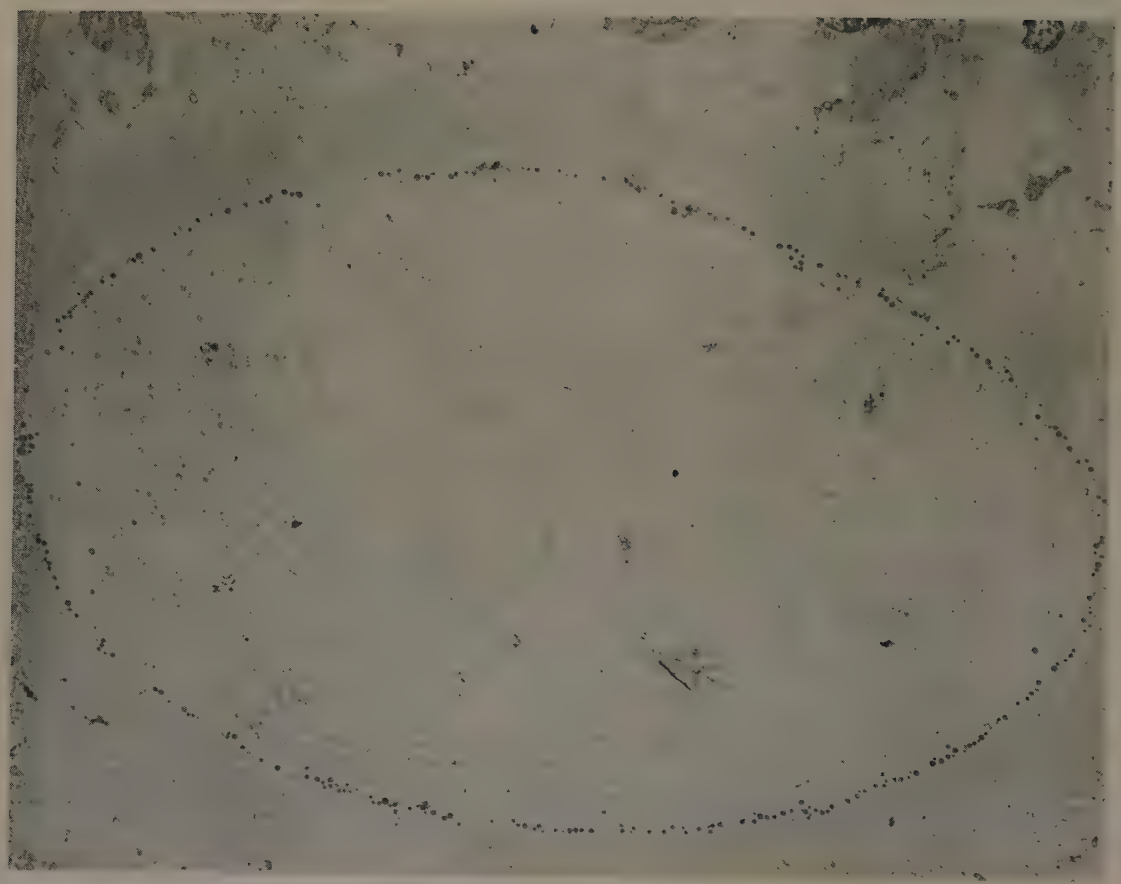

Fig. 6a

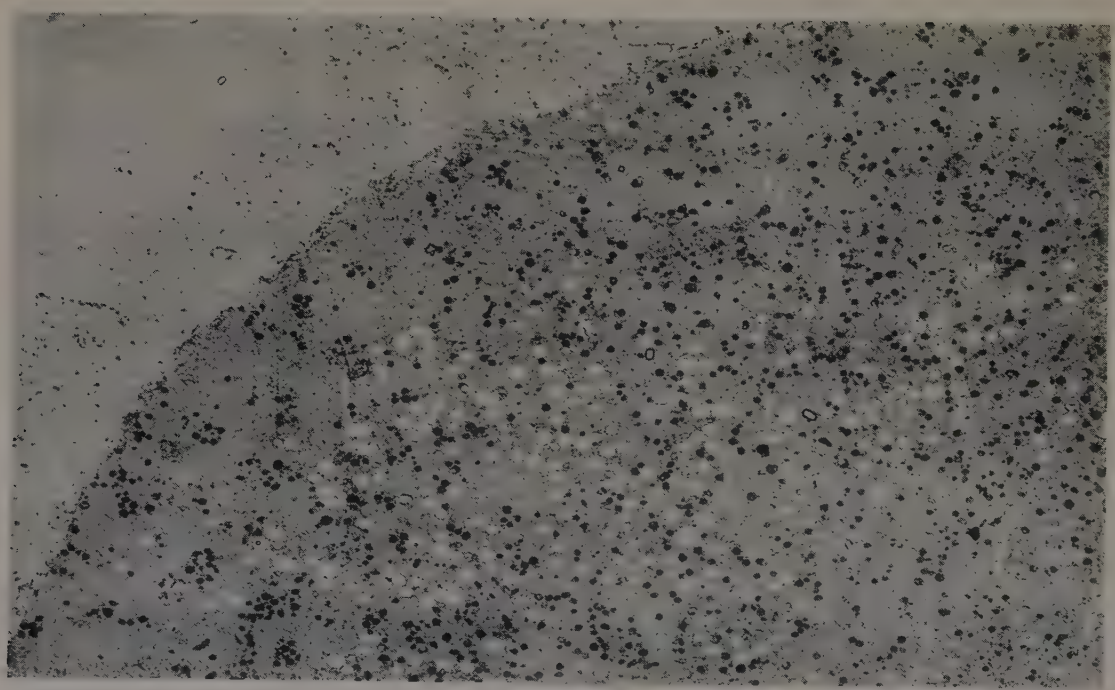

Fig. 6b

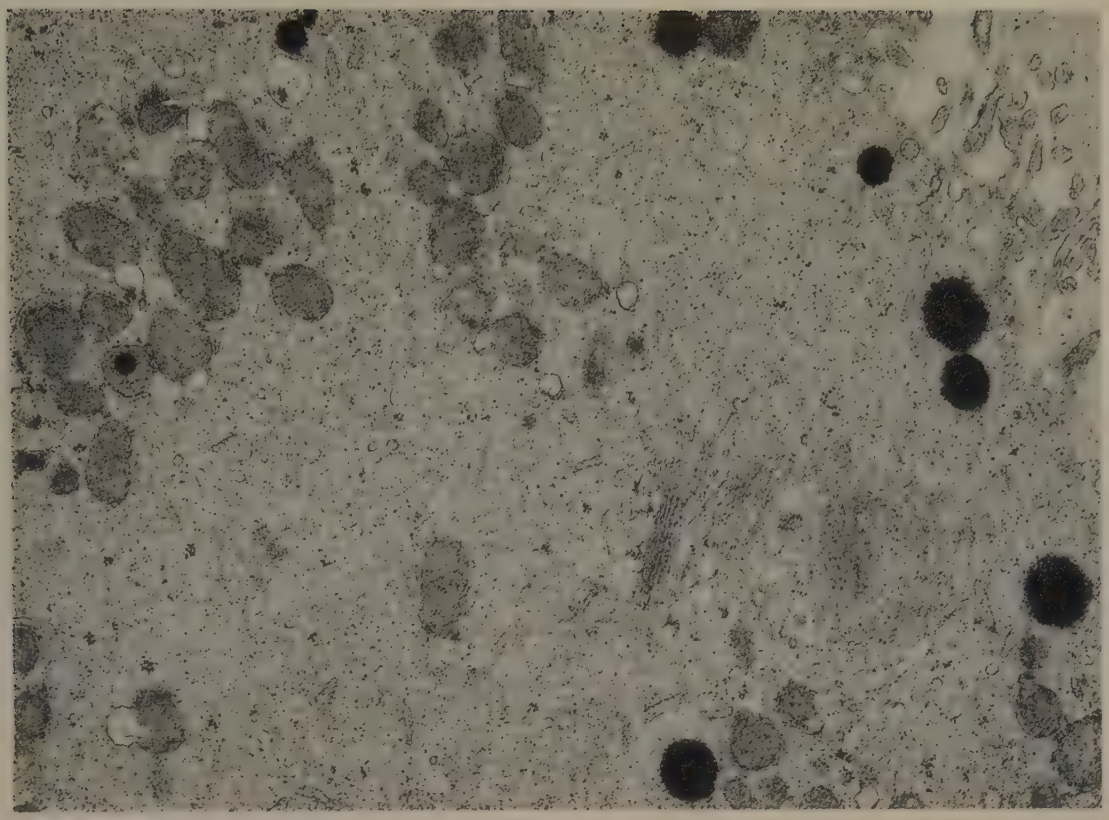

Fig. 7

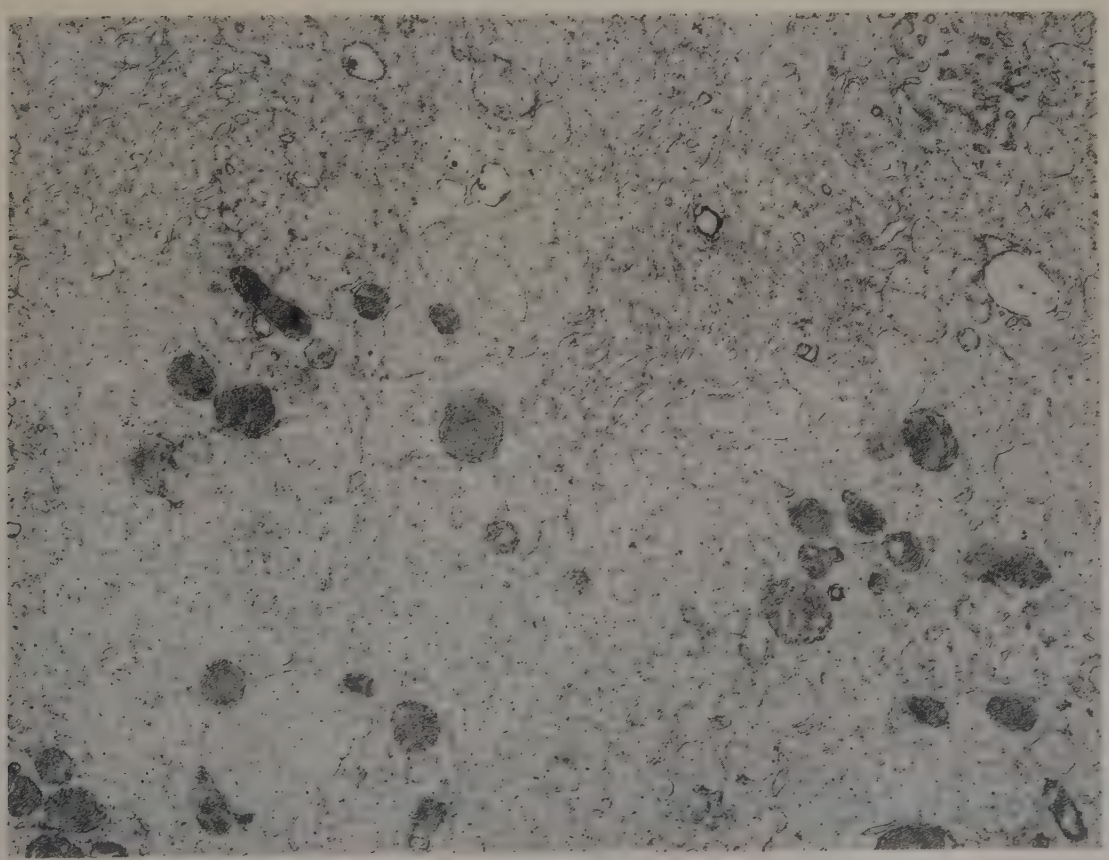

Fig. 8

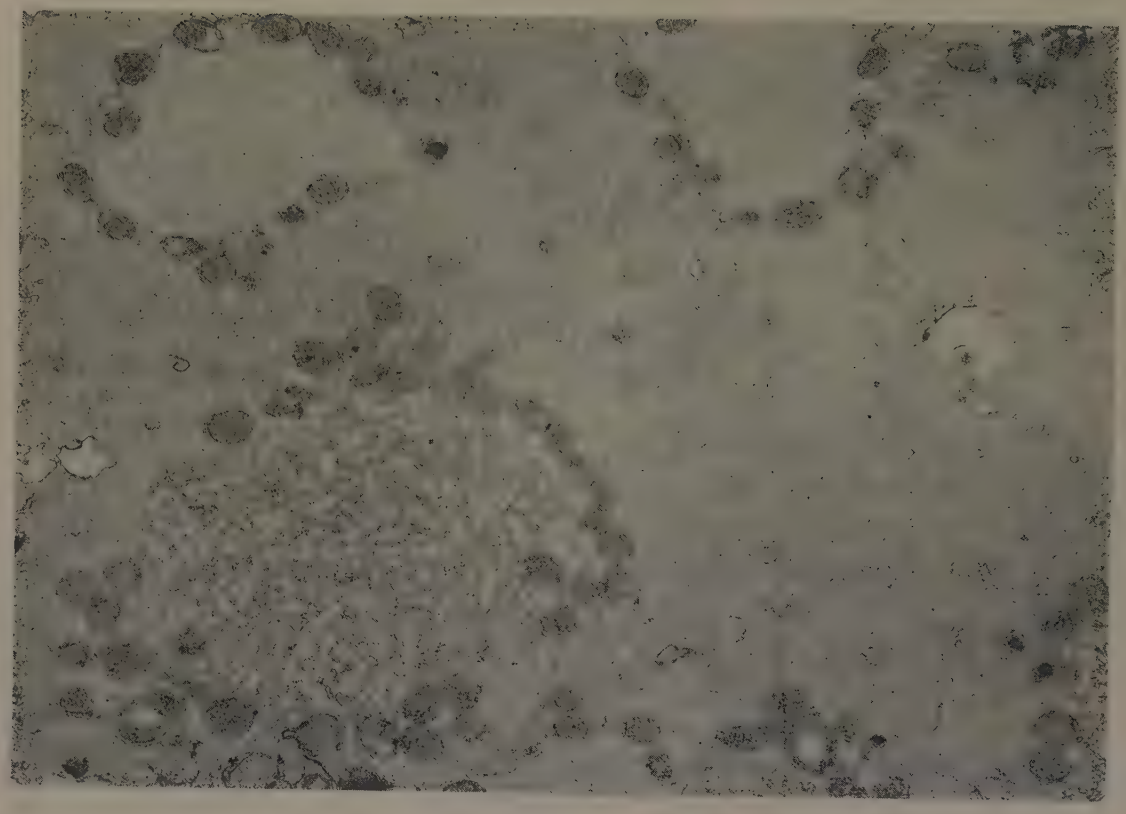

Fig. 9

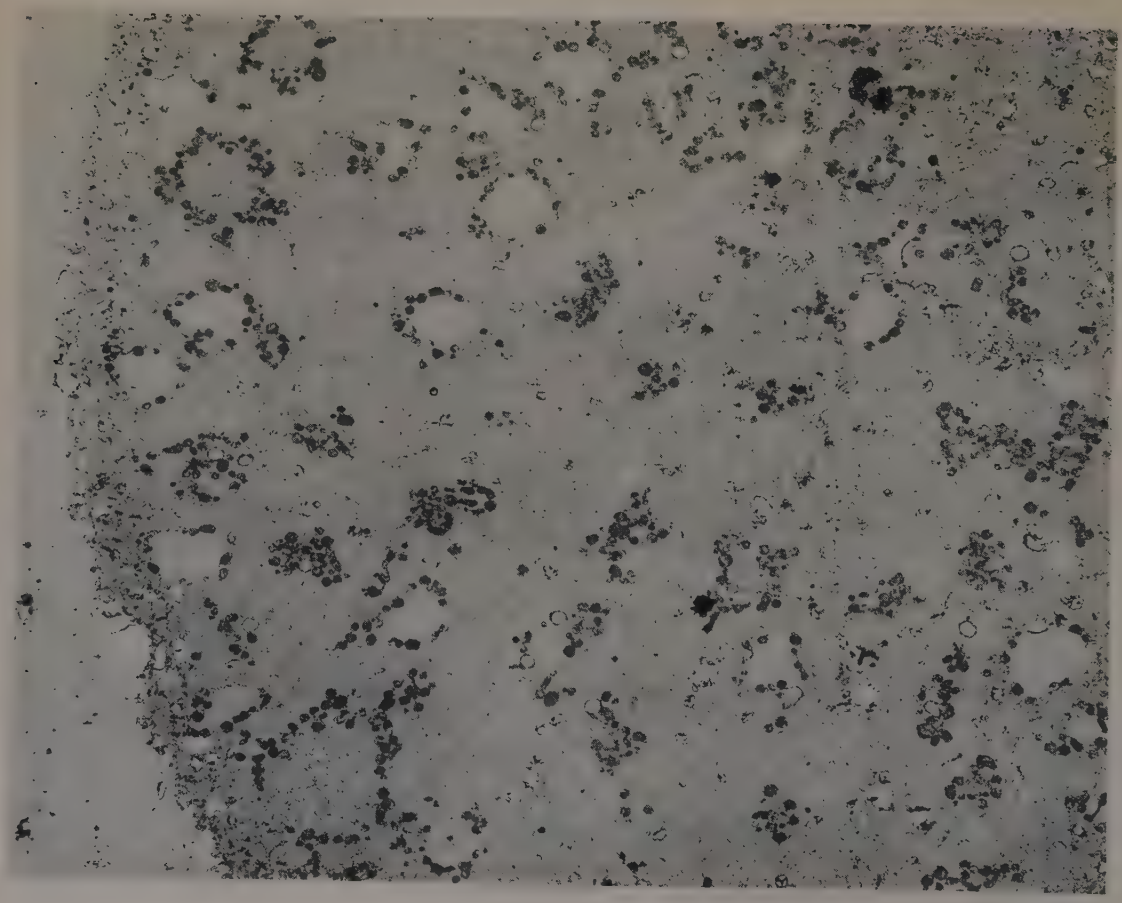

Fig. 10

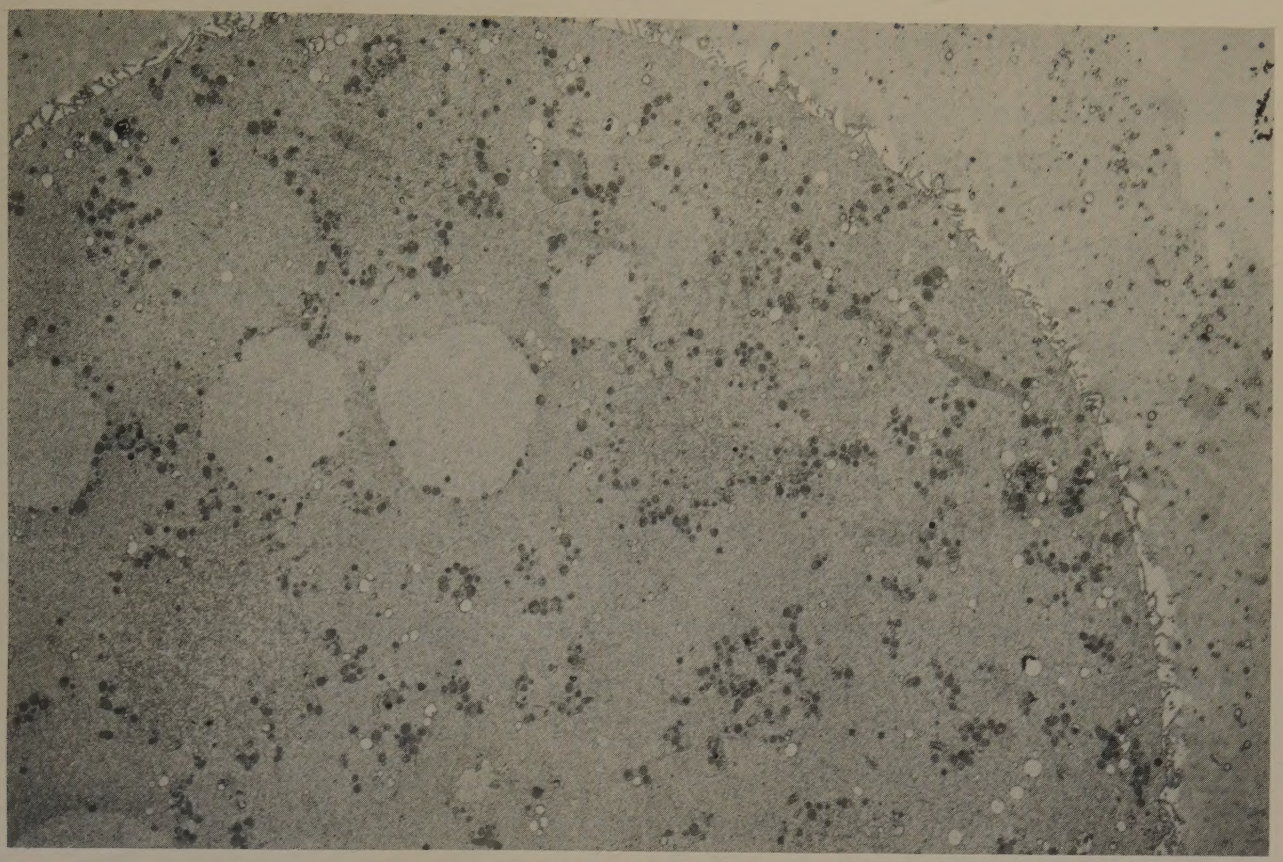

Fig. 11a

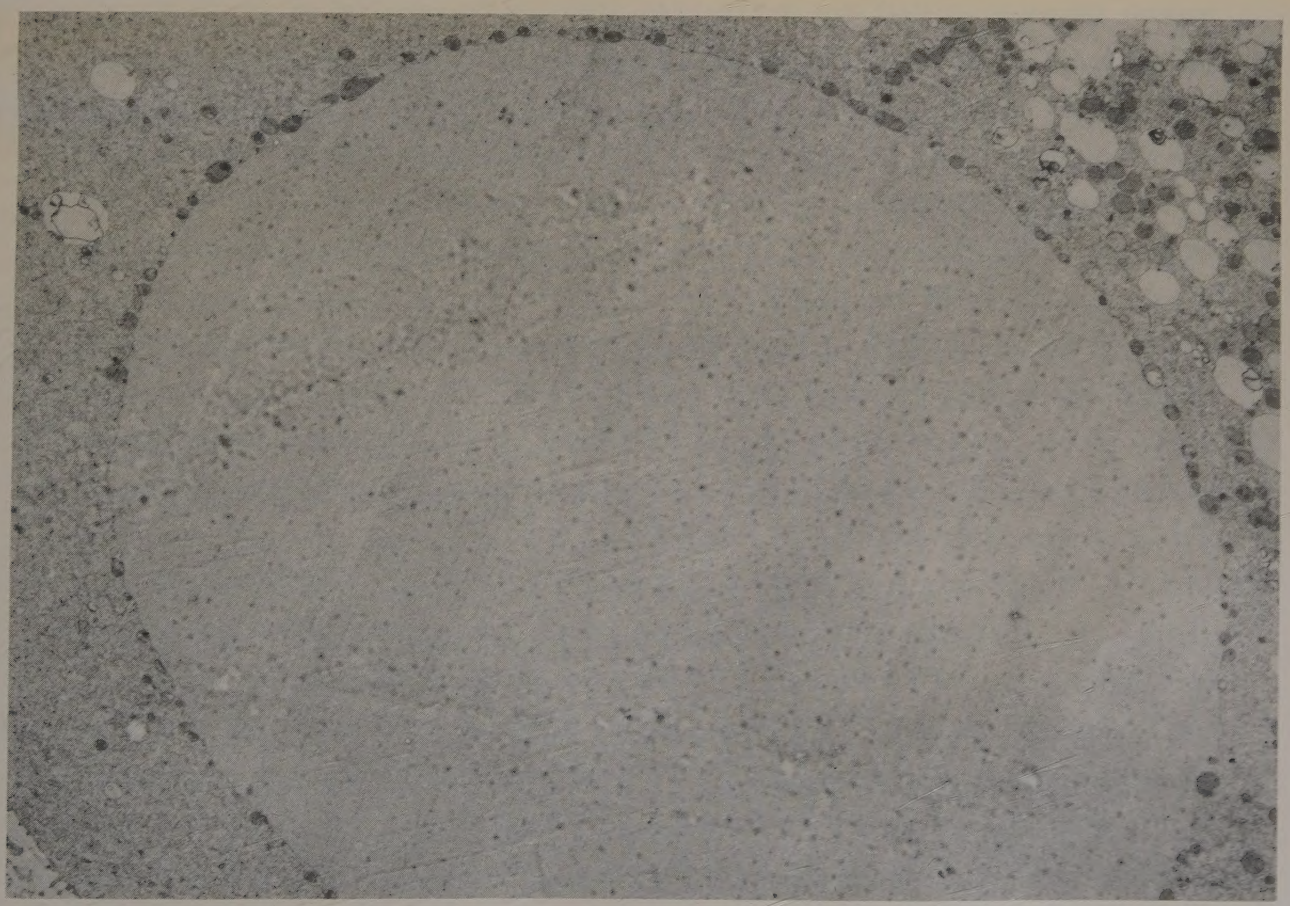

Fig. 11b

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